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Introduction

Nuclear receptors undergo conformational changes when they bind ligands. It should be possible to monitor these changes *in vivo* using energy transfer between fluorophores. The existence of inherently fluorescent proteins such as the variants of jellyfish green fluorescent protein (GFP) suggests that this problem may be approached by making fusions of these proteins to nuclear receptors. We set out to study this problem using the estrogen receptor (ER), a nuclear receptor known to undergo a conformational change upon ligand binding. The proposed assay we have set out to develop is shown in Fig. 1

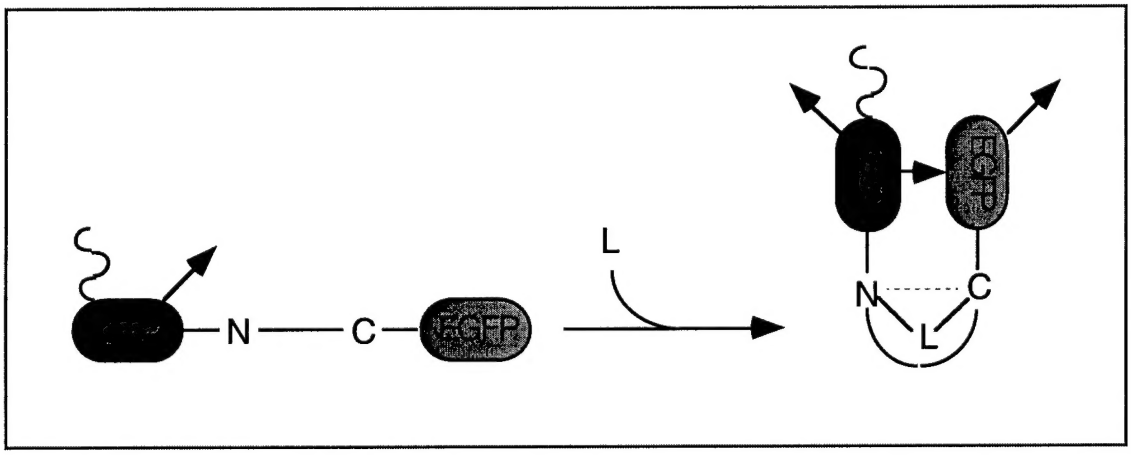

Figure 1: Ligand dependent steroid receptor assay based on FRET detection of conformational changes in the receptor upon hormone binding.

Summary of Progress 2002

Training:

I have gained much needed training in many areas of molecular biology including subcloning, protein expression, transfection of mammalian cell lines, and reporter assays. In addition, I am gaining biochemical training using hormone binding assays.

Technical Objective 1:

Task 1:

I have been unable to generate ligand-dependent FRET. Rather than abandoning the technique which has a lot of promise for steroid receptor research and breast cancer, I have chosen to try to establish the technique using other steroid receptors. I aim to use PR, which is also important in mammary gland development and breast cancer, and AR another steroid receptor for which we have all the necessary clones. We started the additional studies with AR first because the necessary clones were readily available. AR will be a proof of principle receptor for work with PR and ER once we have successfully established FRET.

This last year we switched to making and testing androgen receptor chimeras in order to try and establish ligand-dependent FRET with nuclear receptors. I initially proposed to create estrogen receptor (ER) chimeras with blue fluorescent protein (BFP) and green fluorescent protein (GFP) to generate a novel ligand binding assay based on fluorescence resonance energy transfer (FRET) between the two fluorescent reporters (Figure 1). Owing the failure to detect any ligand-dependent FRET in transfected cells using confocal fluorescent microscopy, we have generated another set of all of the receptor single and double fluorescent chimeras with complimentary fluorescent proteins for the androgen receptor (AR).

Since the analysis about conformation changes of AR has been extensively documented, we wish to use this receptor as an alternative to the ER to establish a

FRET assay and we can investigate why our ER chimeras are unable to produce any positive results in the FRET assays. These pilot experiments would help to establish the basis for constructing the ER-fluorescent fusion protein chimeras that can give positive results in FRET assays. In addition, we are trying to establish chimeras with other receptors, such as the progesterone receptor that are directly relevant to breast cancer. We are trying to check whether the same FRET principle can generally applied to steroid hormone receptors in general. We have then functionally tested all of these AR chimeras in hormone binding and transcription assays. Unfortunately, the results from AR chimeras were similar to what we got from the ER chimeras. Although all of the jellyfish fluorescent protein receptor chimeras bind hormone with an affinity equivalent to that of wild type receptor and were able to transactivate, in a ligand dependent manner, reporter gene expression in transient transfection assays in HeLa cells, the transactivation levels were lower than that observed with wild type AR, suggesting that the fluorescent protein moities may be disrupting the normal interactions of these receptors with coactivators. Similar to the ER chimeras, the AR chimeras were functional in that they bound ligand and activated gene expression.

When we tested the functional fluorescent protein AR chimeras in FRET assays, we encountered similar problems in ER chimeras. We were unable to detect either ligand-dependent or ligand–independent FRET in transfected cells using confocal fluorescent microscopy. Although FRET assays have been reported to use for detection of protein interaction between ER and other proteins, such as SRC-1, FRET can only accomplish when the fluorescent protein is attached to each of the proteins. Up to date, we are still unable to detect any positive FRET signals when we try to establish FRET within a single ER molecule. The fluorescent protein moieties may disrupt the normal dimerization of the N-terminal domain with the ligand-binding domain. In addition, the size of fluorescent protein partners would be so big that it makes two fluorescent proteins be too far apart to engage in FRET. The ligand-dependent conformation changes of ER and AR may not be able to bring two fluorescent proteins close enough for successful FRET.

Objective 1: Development of a PR ligand binding assay using YFP/CFP fluorescent resonance energy transfer.

Task 1: Generation of chimeric receptor fusion gene.

In the light of the technical difficulties to establish a steroid receptor FRET assay. I have changed the focus of the rest of the grant towards this end. Thus, in the upcoming period we will generate PR chimeras. The original SOW is behind schedule due to the technical difficulties establishing the steroid receptor FRET assay. The new SOW is as follows.

Task 2: Assessment of activity of chimeric receptor *in vitro*.

To be completed, dependent on successful completion of task 1.

Task 3: Assessment of activity in transient transfections in cell culture.

To be completed, dependent on successful completion of task 1.

Task 4: Generation and assessment of stable cells with functional YFP:PR:CFP chimeras.

To be completed, dependent on successful completion of task 1.

Objective 2: Testing the PR-based ligand binding assay in vivo by generating transgenic mice expressing the YFP:PR:CFP chimeras from the MMTV promoter.

To be completed, dependent on successful completion of objective 1.

Key Research Accomplishments:

Generation of functional receptor fluorescent protein single and double chimeras with the AR.

Reportable Outcomes:

Manuscript in preparation.